

# Interaction of human Rad51 recombination protein with single-stranded DNA binding protein, RPA

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Received August 10, 1998; Revised and Accepted October 15, 1998

## ABSTRACT

Replication protein A (RPA), a heterotrimeric single-stranded DNA binding protein, is required for recombination, and stimulates homologous pairing and DNA strand exchange promoted *in vitro* by human recombination protein HsRad51. Co-immunoprecipitation revealed that purified RPA interacts physically with HsRad51, as well as with HsDmc1, the homolog that is expressed specifically in meiosis. The interaction with HsRad51 was mediated by the 70 kDa subunit of RPA, and according to experiments with deletion mutants, this interaction required amino acid residues 169–326. In exponentially growing mammalian cells, 22% of nuclei showed foci of RPA protein and 1–2% showed foci of Rad51. After  $\gamma$ -irradiation, the percentage of cells with RPA foci increased to ~50%, and those with Rad51 foci to 30%. All of the cells with foci of Rad51 had foci of RPA, and in those cells the two proteins co-localized in a high fraction of foci. The interactions of human RPA with Rad51, replication proteins and DNA are suited to the linking of recombination to replication.

## INTRODUCTION

Human Rad51 protein (HsRad51) is a homolog of bacterial recombination protein RecA and yeast Rad51 protein (1,2). In addition to a high degree of structural homology to RecA, HsRad51 and yeast Rad51 share significant functional similarity. The proteins form similar nucleoprotein filaments on single-stranded DNA (ssDNA), possess DNA-dependent ATPase activity, and promote homologous pairing and DNA strand exchange between ssDNA and homologous double-stranded DNA (dsDNA) (3–7). The *RAD51* gene of *Saccharomyces cerevisiae* belongs to the *RAD52* epistasis group and, together with other members of the group, is implicated in homologous recombination and repair of DNA double-strand breaks (8). Various studies indicate that mammalian Rad51 protein participates in homologous recombination, DNA repair and immunoglobulin switch recombination (9–14).

Replication protein A (RPA) is a heterotrimeric eukaryotic ssDNA binding protein that is required for the replication, repair

and recombination of DNA. In addition, RPA has been implicated in the regulation of transcription (for review see 15). The subunits of RPA are 70, 32 and 14 kDa in mass. RPA interacts physically with about a dozen other proteins that are involved in replication, repair, recombination and transcription. These interactions are usually mediated by the 70 kDa subunit (15). Yeast and human RPA interact with Rad52, a protein which is involved in homologous recombination (16–18). In *S.cerevisiae*, RPA and Rad52 assemble into subnuclear complexes or foci during meiotic recombination (19); mammalian RPA and Rad51 have recently been shown to co-localize on synapsed axes in meiosis (20). Homologous pairing and DNA strand exchange mediated by yeast or human Rad51 *in vitro* is facilitated by the cognate RPA (4,21–23).

In the course of observations on the polarity of strand exchange promoted by human Rad51 (23), we examined the physical interaction of Rad51 with RPA, as reported here.

## MATERIALS AND METHODS

### Proteins

Human Rad51 protein, human Dmc1 (HsDmc1), human RPA and its mutant forms were purified as described (7,24–26).

### Antibodies

We used polyclonal rabbit sera raised against HsRad51, RecA or HsRPA; affinity purified rabbit polyclonal anti-HsRad51 antibody and monoclonal antibody 7G9 and 71-9A, which recognize the 32 and 70 kDa subunits of HsRPA, respectively. Secondary antibodies (Sigma) used with the polyclonal sera and monoclonal antibodies were goat anti-rabbit conjugated with alkaline phosphatase and goat anti-mouse conjugated with alkaline phosphatase, respectively.

### Co-immunoprecipitation of HsRad51 and RPA proteins

Purified HsRad51 protein (at 0.2–0.9  $\mu$ M final concentration) was incubated with 0.1–0.3  $\mu$ M RPA protein or a mutant form of the protein in 9  $\mu$ l of buffer R (25 mM HEPES, pH 7.4, 1 mM DTT, 100  $\mu$ g BSA/ml) for 30 min at 37°C. Then 1  $\mu$ l anti-HsRad51 affinity-purified rabbit polyclonal antibody (0.7 mg/ml of stock solution) was added. After 30 min incubation at 37°C, we added

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20  $\mu$ l of a 50% slurry of protein A-agarose beads (Pierce Chemical Company), pre-equilibrated in buffer IP (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.02% NP-40). After 60 min incubation at room temperature (with occasional shaking), the beads were washed four times with 400  $\mu$ l buffer IP. The washed beads were mixed with 10  $\mu$ l of loading buffer (100 mM Tris-HCl, pH 7.4, 2% SDS, 20% glycerol, 4 mM EDTA, 200 mM  $\beta$ -mercaptoethanol, 40 mM DTT, 0.08% bromophenol blue), and boiled for 5 min, following which 20  $\mu$ l of the mixture were loaded on a 12% SDS-polyacrylamide gel. After gel electrophoresis, the presence of RPA protein subunits was analyzed by western blotting, using anti-RPA antibodies for specific detection and secondary anti-IgG antibodies conjugated with alkaline phosphatase for visualization.

In control experiments, HsRad51 protein was replaced by an equimolar amount of RecA protein, and anti-RecA antibodies were used for co-immunoprecipitation instead of anti-HsRad51 antibodies.

### Interaction of HsRad51 protein with proteins bound to a nitrocellulose membrane

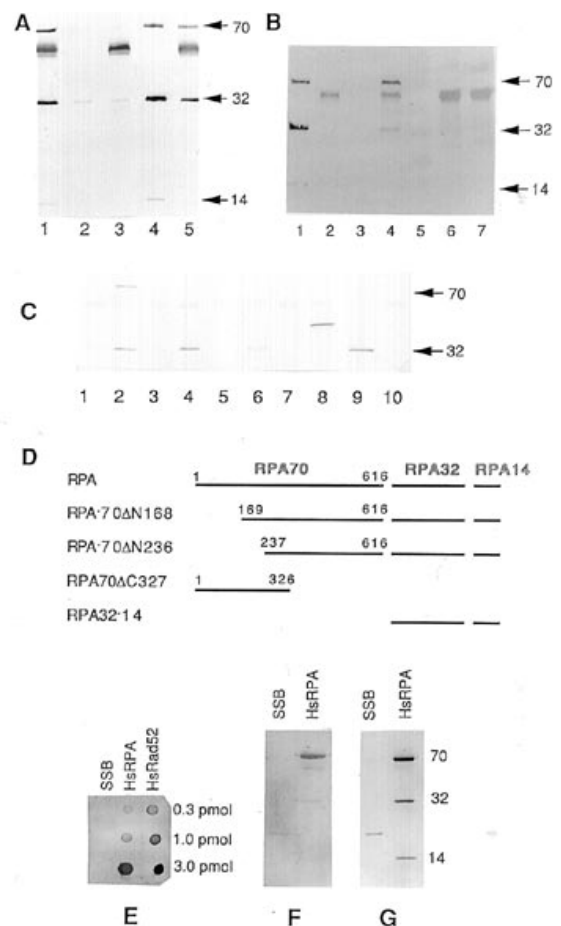
Purified test proteins, diluted in buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50  $\mu$ g BSA/ml) were spotted onto a Biotrace NT nitrocellulose membrane (Gelman Sciences). The membrane was dried for 30 min at room temperature and blocked for 1 h in buffer FW (20 mM Tris-HCl, pH 7.5, 60 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 5% glycerol, 0.02% NP-40), containing 5% non-fat dry milk. After washing in buffer FW, the membrane was incubated for 1 h at room temperature with 0.5 mg HsRad51/ml in buffer FW, containing 2% BSA. The membrane was then washed extensively in buffer FW, and HsRad51 protein that was retained on the membrane was detected by the use of anti-HsRad51 antibodies.

A protocol for far-western blotting was described earlier (27–29). Briefly, proteins were electrophoresed through a 12% SDS-polyacrylamide gel and transferred to Immobilon-P membrane (Millipore Corp.). The membrane was immersed in 8 M urea and 1% mercaptoethanol in buffer FW. Proteins adsorbed to the membrane were renatured by incubation in 10 sequential 2-fold dilutions of urea in buffer FW. After blocking in buffer FW containing 5% non-fat dry milk, the membrane was incubated for 1 h at room temperature with 0.5 mg HsRad51 protein/ml in buffer FW containing 2% BSA. The bands which retained HsRad51 were detected using anti-HsRad51 antibodies.

## RESULTS

### Co-immunoprecipitation of RPA and HsRad51

Using co-immunoprecipitation to test for physical interactions, we mixed HsRad51 and RPA prior to adding purified anti-HsRad51 antibodies and binding immune complexes to beads of protein A-agarose. The material that was retained by thoroughly washed beads was recovered by boiling in SDS and was analyzed by gel electrophoresis and western blotting with anti-RPA antibodies (Fig. 1). All three subunits of RPA were found in the immune complexes retained on beads of protein A-agarose (Fig. 1A, lane 1). Retention of RPA required the presence of both HsRad51 and anti-HsRad51 (Fig. 1A, lanes 2 and 3). Under our conditions, almost all HsRad51 was retained by washed beads (data not shown), and we estimate that ~10% of RPA was retained.



**Figure 1.** Physical interactions of HsRad51 and RPA. (A) Co-immunoprecipitation. RPA protein at 0.3  $\mu$ M final concentration was incubated with 0.9  $\mu$ M HsRad51 (lanes 1 and 2) or HsDmc1 (lane 5). Anti-HsRad51 antibody was used for immunoprecipitation; anti-RPA antibody was used for detection. Lane 2, no anti-HsRad51 antibodies were added. Lane 3, HsRad51 protein was omitted from the incubation mixture. Lane 4, 35 ng of RPA were used to provide molecular weight markers. (B) Specificity of co-immunoprecipitation. RPA (at 0.1  $\mu$ M final concentration) was incubated with 0.2  $\mu$ M HsRad51 (lanes 3 and 4) or RecA (lane 7). Anti-Rad51 (lanes 2 and 4) or anti-RecA (lanes 6 and 7) antibodies were used for immunoprecipitation; anti-RPA was used for detection. Lane 2, HsRad51 was omitted. Lane 6, RecA was omitted. Lane 3, no anti-HsRad51 antibodies were added. Lane 5, Rainbow molecular weight markers (Amersham) were used to monitor the transfer from gel to membrane. Lane 1, 20 ng of RPA was used to provide molecular weight markers. (C) Co-immunoprecipitation, location of sites of interaction. RPA protein or its mutated forms (at 0.3  $\mu$ M final concentration) were incubated with 0.9  $\mu$ M HsRad51 (lanes 2, 4, 6, 8 and 9). Anti-HsRad51 antibodies were used for immunoprecipitation. Lanes 1 and 2, RPA protein; lanes 3 and 4, mutant RPA-70 $\Delta$ N168; lanes 5 and 6, mutant RPA-70 $\Delta$ N236; lanes 7 and 8, mutant RPA70 $\Delta$ C327; lanes 9 and 10, mutant RPA32-14. Lanes 1, 3, 5, 7 and 10, HsRad51 was omitted. Anti-RPA antibodies were used to detect RPA which co-precipitated with HsRad51. Numbers at the right of (A), (B) or (C) indicate molecular weights of RPA subunits in kDa. (D) Schematic diagrams of RPA mutants. Beginning and ending amino acids of each mutant are indicated. (E) Binding of HsRad51 protein to RPA immobilized on membranes. Equimolar amounts of the proteins shown were spotted on a nitrocellulose membrane; numbers at the right indicate the amount of those proteins spotted. The membrane was incubated with HsRad51 protein whose retention on the membrane was detected using anti-HsRad51 antibodies. (F) Far-western blotting. About 15 pmol of RPA and SSB were separated on an SDS-polyacrylamide gel and transferred to a membrane. After protein renaturation, the membrane was incubated with HsRad51 and washed extensively. HsRad51 protein that was retained by the membrane was detected by anti-HsRad51 antibodies. (G) Coomassie Blue stained gel of proteins run in parallel. Numbers at the right indicate molecular weights of RPA subunits in kDa.

Polyclonal antibody against HsRad51 cross-reacts with HsDmc1, a RecA homolog that is specific to meiotic cells (data not shown). The substitution of HsDmc1 for HsRad51 also resulted in co-immunoprecipitation of RPA (Fig. 1A, lane 5).

As one test of the specificity of the interactions with RPA, we substituted *Escherichia coli* RecA protein for HsRad51 and anti-RecA antibody for anti-HsRad51 antibody in the immunoprecipitation. Although antibody to RecA led to almost complete adhesion of RecA to protein A-agarose beads (data not shown), co-immunoprecipitation of RPA by RecA was undetectable (Fig. 1B, lane 6).

The ability of RPA to bind to HsRad51 protein was confirmed by experiments in which RPA was immobilized on a nitrocellulose membrane and subsequently incubated with HsRad51 protein. Retention of the HsRad51 on the washed membrane was analyzed using anti-HsRad51 antibody. As a negative control, we used *E. coli* ssDNA binding protein (SSB), and as a positive control we used human Rad52 protein, which has been observed to bind to HsRad51 protein (30). By this assay, HsRad51 bound to RPA and to HsRad52, but not to *E. coli* SSB (Fig. 1E).

Since both HsRad51 and RPA bind to ssDNA, it was necessary to rule out the possibility that the observed HsRad51–RPA association was mediated by contaminant DNA in the enzyme preparations. When we applied the protocol for labeling 3' termini of DNA by terminal transferase (31), we failed to detect any radioactive DNA (<1 pM) in the enzyme preparations that were used in our experiments.

#### Interaction with HsRad51 is mediated by the 70 kDa subunit of RPA

The interaction of RPA and HsRad51 was further analyzed by the far-western protocol. Equimolar amounts of purified RPA and SSB proteins were electrophoresed through SDS–polyacrylamide gel and transferred to a membrane. Proteins bound to the membrane were renatured *in situ* by incubation of the membrane in solutions with gradually decreasing concentrations of urea. The membrane was incubated with a solution of HsRad51 protein, and following extensive washing, any retained HsRad51 was detected by use of anti-HsRad51 antibodies. As indicated in Figure 1F, HsRad51 bound to the 70 kDa subunit of RPA protein. Binding was not detected to the other subunits of RPA nor to *E. coli* SSB. This observation suggests that the interaction of HsRad51 with RPA is mediated by the largest subunit of RPA.

#### Mapping the interacting domain of the 70 kDa subunit of RPA

To locate the domain of the 70 kDa subunit that interacts with HsRad51, we examined co-immunoprecipitation of HsRad51 with mutant forms of HsRPA (Fig. 1D; 26). We found that both mutant RPA-70ΔN168, which has lost 168 amino acids from the N-terminus of the 70 kDa subunit, and mutant RPA70ΔC327, which consists of 326 N-terminal amino acids of the 70 kDa polypeptide and which lacks the 32 and 14 kDa subunits, co-immunoprecipitated with HsRad51 (Fig. 1C, lanes 4 and 8). On the other hand, mutant RPA-70ΔN236, which has lost 236 amino acid residues from its N-terminus, did not co-immunoprecipitate efficiently with HsRad51 (Fig. 1C, lane 6). These observations indicate that residues between 168 and 327 of the 70 kDa subunit of the RPA are important in the trimeric protein for interaction

with HsRad51. The same domain has been implicated in interactions with several other proteins (26).

The ability of mutant RPA70ΔC327 to interact with HsRad51 indicates that such interaction does not require 32 and 14 kDa subunits. Furthermore, mutant RPA-70ΔN236, which carries intact 14 and 32 kDa subunits and a deleted form of the 70 kDa subunit, does not interact with HsRad51. Nevertheless, we found that mutant RPA32-14, which consists of only the 14 and 32 kDa subunits, co-immunoprecipitated with HsRad51 (Fig. 1C, lane 9). This additional interaction with the 32 or 14 kDa subunit was apparent only when the 70 kDa subunit was absent from the trimeric complex.

#### Co-localization of HsRad51 and RPA proteins in mammalian cells

The finding of a direct interaction between HsRad51 and RPA proteins *in vitro* prompted us to compare the intracellular localization of the two proteins. Consistent with our earlier observations (10), we found that HsRad51 protein concentrated in multiple discrete foci in the nucleoplasm of cultured mouse fibroblasts, although only in 1% of cells. However, after  $\gamma$ -irradiation of cells with doses of 5–10 Gy, the number of cells with focally concentrated HsRad51 protein increased to >30% (Table 1). In the untreated exponentially growing cultures, 22% of nuclei showed focally concentrated RPA protein, whereas the majority of cells displayed a more or less uniform nuclear immunofluorescence of weak to moderate intensity. After  $\gamma$ -irradiation, the percentage of cells with RPA foci increased to >50%. Thus the absolute increases in the fraction of HsRad51-positive cells and RPA-positive cells were similar, and virtually all of the cells with foci of HsRad51 had foci of RPA, both of which were attributable to irradiation (Table 1). Moreover, within 24 h after irradiation, HsRad51 and RPA co-localized in most of the cells that had foci of both proteins (Table 1 and Fig. 2). This association between HsRad51 and RPA occurred in a time-dependent manner. The highest number of HsRad51-positive cells as well as the highest number of co-localizations were observed 1 day after DNA damage. After 2 days, the number of HsRad51 positive cells showing co-localization with RPA foci dropped from nearly 100 to 50% (Table 1).

Additional observations indicated that focal co-localization of HsRad51 and RPA was mediated by DNA damage. Focal concentration of HsRad51 was also induced by treatment of cell cultures with the aneuploidogen colcemid, which does not introduce significant DNA damage (T.Haaf, unpublished observations), but these colcemid-induced HsRad51 foci did not co-localize with RPA foci (data not shown).

We conclude that  $\gamma$ -irradiation leads to the concentration of HsRad51 and RPA in common foci.

#### DISCUSSION

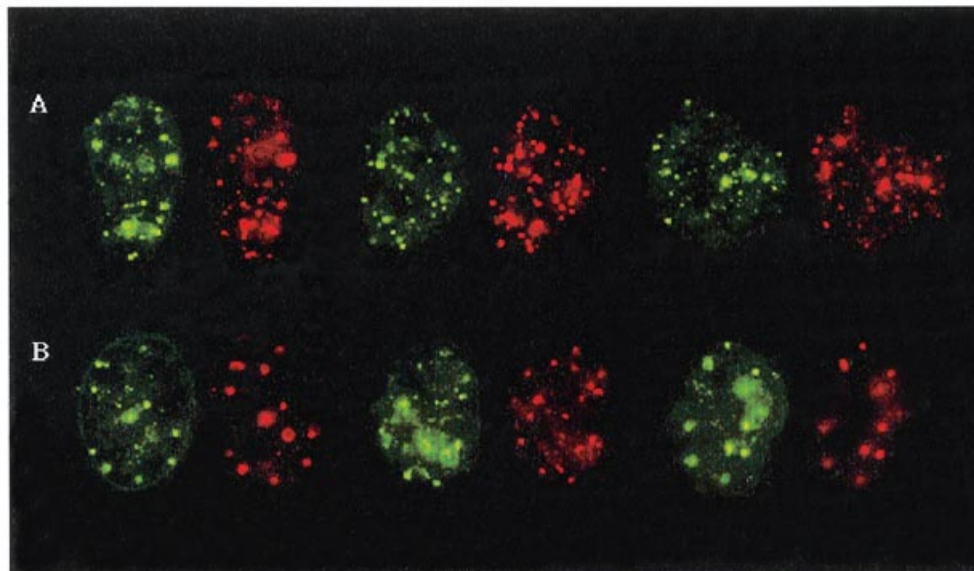
SSB, the ssDNA-binding protein from *E. coli*, is an essential protein that plays crucial roles in DNA replication, DNA repair and homologous recombination. This protein removes secondary structure from ssDNA, and by doing so facilitates the activity of a variety of enzymes of DNA metabolism (for review see 32).

RPA, the eukaryotic counterpart of the SSB, is also implicated in various aspects of cellular metabolism. Unlike SSB, which is composed of identical 20 kDa subunits, RPA is a stable heterotrimeric complex of 70, 32 and 14 kDa subunits. Its impact

**Table 1.** Co-localization of HsRad51 and HsRPA in nuclear foci after  $\gamma$ -irradiation

|                              | HsRad51-negative cells |                | HsRad51-positive cells |                | HsRad51-positive cells showing co-localization of HsRad51 and HsRPA |
|------------------------------|------------------------|----------------|------------------------|----------------|---|
|                              | HsRPA negative         | HsRPA positive | HsRPA negative         | HsRPA positive |   |
|                              | %                      | %              | %                      | %              | %   |
| <u>SMZ mouse fibroblasts</u> |                        |                |                        |                |   |
| No treatment                 | 78                     | 21             | 0                      | 1              | n.d.  |
| 6 h after 5 Gray             | 47                     | 18             | 1                      | 34             | 77  |
| 6 h after 10 Gray            | 41                     | 21             | 2                      | 36             | 84  |
| 24 h after 5 Gray            | 46                     | 21             | 1                      | 32             | 97  |
| 48 h after 5 Gray            | 52                     | 32             | 1                      | 15             | 43  |
| 48 h after 10 Gray           | 46                     | 18             | 4                      | 32             | 54  |
| <u>TGR rat fibroblasts</u>   |                        |                |                        |                |   |
| 24 h after 5 Gray            |                        |                |                        |                | 73  |

At least 300 cells were analyzed for each experiment. The numbers in the first four columns represent the distribution of all cells examined in each experiment. HsRad51-positive and HsRPA-positive cells have either >10 foci of medium immunofluorescence intensity or some very bright foci. The last column shows the percentage of HsRad51-positive cells, in which  $\geq 30\%$  of HsRad51 foci also contained focally concentrated HsRPA.



**Figure 2.** Co-localization of HsRad51 and RPA in cultured mouse fibroblasts following  $\gamma$ -irradiation. Mouse fibroblasts 6 (A) and 24 h (B) after  $^{60}\text{Co}$  irradiation with a dose of 5 Gy. Images of nuclei are arranged in pairs in which the one on the left shows staining by anti-HsRad51 (green), and the one on the right staining of the same nucleus by anti-RPA (red). Both HsRad51 and RPA are concentrated in discrete nuclear foci which co-localized in a high percentage of HsRad51-positive cells (Table 1). In addition, both proteins produce a more or less uniform or fine punctate staining of the entire nucleus.

on the replication, repair and recombination of DNA can be explained only partly by its role in removing the secondary structure of ssDNA, since it also interacts with a variety of proteins through which it exerts its vital effects (reviewed in 15).

Using co-immunoprecipitation, we found that RPA interacts with HsRad51 as well as with Dmc1, its meiotic homolog. The interaction of RPA with HsRad51 was also confirmed by a blotting assay. As expected, RPA did not co-immunoprecipitate with RecA, and according to blotting experiments, *E.coli* SSB protein did not interact with Rad51. At a ratio of two to three molecules of HsRad51 per molecule of RPA heterotrimer, the complete precipitation of HsRad51 was accompanied by the

precipitation of only 10% of the input RPA. This apparently weak interaction of RPA with HsRad51 was also reflected in an ELISA which indicated that the interaction of RPA with HsRad52 was stronger than that with Rad51 (data not shown).

In experiments with mutant forms of RPA protein, we found that a region of the 70 kDa subunit of RPA that included residues 169–326 is necessary for the interaction of RPA with Rad51. The same domain of the 70 kDa subunit has been implicated previously in the interactions of RPA with several other proteins (26).

In the  $G_1$ -phase of the cellular cycle, RPA is dispersed throughout nuclei, as shown by immunostaining. By contrast, in 60–70% cells in S-phase, RPA is rearranged in a punctate pattern,

or in a mixed punctate and dispersed pattern. The punctate appearance of RPA in S-phase is presumably due to its localization in replication foci (33–38). In agreement with the observations just cited, we found focally concentrated RPA in 22% of non-synchronized cells. We assume that those foci represent sites where chromosomal replication takes place during S-phase. After  $\gamma$ -irradiation, however, the percentage of cells with RPA foci was increased to 52%. Earlier we had found that after DNA damage HsRad51 protein changes its nuclear distribution and moves to distinct areas, forming foci (10). Recently, we found that the majority of these foci contained a region of ssDNA, which appeared in cells as a result of DNA damage. We postulate that these foci are nucleated by ssDNA where Rad51 forms filaments and mediates recombinational repair (E.Raderschall, E.I.Golub and T.Haaf, submitted for publication). The observations reported here show that these damage-induced Rad51 foci also contain RPA and thus provide further evidence for a role of RPA in recombinational repair. Mammalian RPA and Rad51 have also been shown to co-localize on synapsed axes in meiosis, suggesting an interaction of RPA with Rad51 in meiotic recombination (20).

In the recombination system of *S.cerevisiae*, RPA has been shown to stimulate homologous pairing and strand exchange promoted by Rad51 (21,22). However, RPA actually inhibits the reaction unless it is added after Rad51 has formed a filament on ssDNA (39,40). Other observations suggested that RPA acts by removing secondary structure from ssDNA, an interpretation supported by the further observation that *E.coli* SSB could replace RPA in reactions with Rad51 (22). This non-specific aspect of the action of RPA, however, does not exclude other specific interactions. Rad52, which interacts *in vivo* with Rad51 (41), is able to overcome the inhibition caused by RPA if the Rad51 filament has not already been formed (39,40,42). Rad52 is also able to promote the annealing of complementary strands (43). RPA, which interacts with Rad52 *in vivo* (16), enables Rad52 to promote the annealing of kb-lengths of ssDNA, and in this function, neither human RPA nor *E.coli* SSB can replace yeast RPA (44).

Human RPA also stimulates pairing and strand exchange by human Rad51, but the nature of this interaction is less clear than in the case of the yeast enzymes (23,45).

In both prokaryotes and eukaryotes, 3' ends play a favored role in the initiation of recombination (46–49). According to the double-strand break model of recombinational repair, an invading 3' end provides a primer for new DNA synthesis that is important in completing the repair (50). A similar mechanism operates in late phase T4 replication when recombination initiates the formation of replication forks (51), and a similar process is thought to occur in the repair of broken replication forks by recombination (52–54). Moreover, in mammals, Rad51 plays a vital role as evidenced by the embryonic lethality of homozygous Rad51 null mutations (11,55). Human RPA interacts specifically with viral proteins that initiate DNA replication, such as SV-40 large T-antigen, EBNA1 protein from Epstein–Barr virus and E2 protein from bovine papilloma virus (56–58), as well as with cellular DNA polymerase  $\alpha$  and the primase subunit (26,56). The 70 kDa subunit of RPA also interacts with mammalian PCNA, the toroidal sliding clamp of replication (59). The present demonstration of a physical interaction between RPA and Rad51, as well as their co-localization in  $\gamma$ -irradiated cells, is consistent with the hypothesis that RPA

creates a bridge between recombination and replication in mammalian cells (23).

## ACKNOWLEDGEMENTS

We are grateful to Jan Zulkowski for data processing and to Zhufang Li for technical assistance. This research was supported by Grant no. R37-GM33504 from the National Institutes of Health to C.M.R., Research Grant HA1374/4-1 from Deutsche Forschungsgemeinschaft to T.H. and Grant no. GM44721 to M.S.W.

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